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**Human blue cone opsin regeneration involves secondary retinal binding with analog specificity**

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**Running title:** *Blue cone opsin regeneration*

**Abbreviations:** 9CR; 9-*cis*-retinal, 11CR; 11-*cis*-retinal, ATR; all-*trans*-retinal, DDM; n-dodecyl- $\beta$ -D-maltoside, POPC; 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphatidylcholine, SB; Schiff base, TM; transmembrane helix

**Keywords:** color vision, G protein-coupled receptor, retinoid, ligand binding, allostery, spectroscopy

## **Abstract**

Human color vision is mediated by the red, green and blue cone visual pigments. Cone opsins are G protein-coupled receptors consisting of an opsin apoprotein covalently linked to the 11-*cis*-retinal chromophore. All visual pigments share a common evolutionary origin and red and green cone opsins exhibit a higher homology, whereas blue cone opsin shows more resemblance to the dim light receptor rhodopsin. Here we show that chromophore regeneration in photoactivated blue cone opsin exhibits intermediate transient conformations and a secondary retinoid binding event with slower binding kinetics. We also detected fine-tuning of the conformational change in photoactivated blue cone opsin binding site that alters the retinal isomer binding specificity. Furthermore, the molecular models of active and inactive blue cone opsins show specific molecular interactions in the retinal binding site which are not present in other opsins. These findings highlight the differential conformational versatility of human cone opsin pigments in the chromophore regeneration process, particularly compared to rhodopsin, and point to relevant functional unexpected roles other than spectral tuning for the cone visual pigments.

## INTRODUCTION

Human color vision is mediated by the red, green and blue cone photoreceptor cells. Blue cones represent ~10% of all cone photoreceptor cells and are responsible for short-wavelength photopic vision. These cells are distributed unevenly throughout the retina, unlike the red/green photoreceptor cells that are populated mainly in the fovea (1,2). The outer segment of these photoreceptor cells contains densely packed light-absorbing receptors, the blue cone opsins. Blue cone opsin shares 46% sequence similarity with rhodopsin, the well characterized visual pigment from rod photoreceptor cells specialized in dim-light scotopic vision (3). Opsins are seven transmembrane (TM) helical G protein-coupled receptors (GPCRs), bound to an 11-*cis*-retinal (11CR) chromophore in its dark state (inactive) conformation. Photon absorption (short wavelength, ~420 nm, photons in the case of blue cone opsin) triggers the isomerization of 11CR to all-*trans*-retinal (ATR) and starts a series of conformational changes that lead to active MetarhodopsinII intermediate (MetaII). MetaII conformation, with bound ATR, gradually decays and the retinal eventually leaves the protein binding pocket (4,5). MetaII is the functionally-active conformation and its interaction with the G-protein, transducin, initiates the visual signal transduction pathway (6). MetaII should be eventually converted back to the dark-adapted state by regeneration with 11CR for receptor turn-over but the structural details of such conformational rearrangements are still unclear. MetaII intermediate of blue cone opsin decays in less than a second when compared to the decay time of about 10 min for rhodopsin (7,8). Such a fast decay of its active intermediate requires rapid adaptation in order to maintain a sufficient supply of regenerated dark-adapted blue cone opsins which can ensure an appropriate response to a continuous bright light stimulus (9). The detailed molecular mechanism behind the regeneration of free opsin is unclear, but these cone photoreceptor cells have been shown to engage in a different chromophore recycling pathway which is 20-fold faster than the canonical pigment epithelium pathway of rod photoreceptor cells (10).

Lys293<sup>7,43</sup> at TM7 in blue cone opsin (superscripts refer to the general numbering system for GPCRs (11)), is the site of covalent attachment for 11CR via a protonated Schiff base (SB) linkage. The protonated SB is stabilized by Glu110<sup>3,28</sup> at TM3 that acts as the counterion (12,13), though SBs from other short wavelength opsins are unprotonated (14). Without any perturbation, bare protonated SB shows a maximum absorbance at 440 nm and the 12 specific amino acids, from blue cone opsin, responsible for the spectral shift to 420 nm have been previously identified. (15,16). In dark, the retinal  $\beta$ -ionone ring is located close to Tyr262<sup>6,48</sup> (Trp281<sup>6,48</sup> or Trp265<sup>6,48</sup> in red/green cone opsin and rhodopsin, respectively). Upon photoactivation, the fluorescence of this aromatic residue (with the potential contribution of Trp123<sup>3,41</sup>, Phe205<sup>5,43</sup> and Phe209<sup>5,47</sup>) is no longer quenched by the retinal molecule causing a fluorescence increase in the environment which has been used to study the changes at the retinal binding site of opsins by fluorescence spectroscopy (17,18).

We have previously compared the regeneration process of photoactivated rhodopsin and red cone opsin *in vitro*, and identified key differences in the regeneration mechanism, with retinal analogs. This implies that red cone opsin has its binding pocket in a relatively more “open” conformation compared to rhodopsin (18). These findings provided novel insights into the precise fine-tuning mechanism of ligand binding to GPCRs (19). In another recent study, we found differences in SB protonation in the transient intermediates for green cone opsin when compared to red cone opsin upon regeneration. Moreover, we proposed the existence of a secondary retinal binding site for red and green cone opsins (20). In a parallel approach, here we have analyzed the regeneration mechanism of blue cone opsin pigment with two retinal analogs. Our results can be interpreted as reflecting structural differences in the regeneration process of photoactivated blue cone opsin compared to those of red and green cone opsins. Such differences may have been relevant in the evolutionary course of color vision development in vertebrates.

## **MATERIALS AND METHODS**

### **Materials**

The green and blue cone opsin genes, cloned into pMT4 plasmid vector, were kindly provided by Prof. Kevin D. Ridge. Dulbecco’s modified Eagle medium (PAA Laboratories), supplemented with fetal bovine serum (Sigma), L-glutamine (Sigma), and penicillin-streptomycin (Sigma), was used to culture COS-1 cells (American Type Culture Collection no. CRL-1650). 11CR was provided by the National Eye Institute, National Institutes of Health. Purified mAb rho-1D4 was obtained from Cell Essentials and was coupled to CNBr-activated Sepharose beads (Sigma). n-dodecyl-b-D-maltoside (DDM) was purchased from Affymetrix. The nonamer-peptide H-TETSQVAPA-OH was obtained from Unitat de Tècniques Separatives i Síntesi de Pèptids, Universitat de Barcelona. 9CR, hydroxylamine, protease inhibitor cocktail, and phenylmethanesulfonyl fluoride (PMSF) were purchased from Sigma, and polyethyleneimine (PEI) was purchased from Polysciences. Quartz absorption cuvettes were from Hellma Analytics (Germany).

### **Expression and purification of cone pigments**

Cone opsins were expressed in transiently transfected COS-1 cells by chemical transfection using PEI reagent. Cells were harvested 48–60 h after transfection and regenerated with 10  $\mu$ M 11CR in PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na<sub>2</sub>HPO<sub>4</sub>, and 1.8 mM KH<sub>2</sub>PO<sub>4</sub>), pH 7.4, by overnight incubation at 4°C. Regenerated cells were subsequently solubilized using 1% DDM with PMSF and protease inhibitors, and the pigments were purified by immunoaffinity

chromatography using Sepharose 4B coupled to rho-1D4 antibody. The bound cone opsins were eluted in PBS buffer containing the nonamer-peptide and 0.05% DDM.

### **Characterization of blue cone opsin by means of UV-visible spectrophotometry**

Purified cone pigment was spectroscopically characterized using a Varian Cary 100 Bio spectrophotometer (Varian), equipped with a water-jacketed cuvette holder connected to a circulating water bath. Temperature was controlled by a peltier accessory connected to the spectrophotometer and the temperature was maintained at 20°C during all the experiments. All the spectra were recorded in the 250 nm – 650 nm range, with a bandwidth of 2 nm, a response time of 0.5 s, and a scan speed of 400 nm/min.

#### *Pigment acidification*

The presence of retinylidene Schiff base in visual pigments can be measured by acidifying the sample using 2 M H<sub>2</sub>SO<sub>4</sub>, to pH 1.9 (21). In dark state, acidification disrupts the spectral tuning arising from retinal and opsin interactions but not the protonated SB which shifts the absorbance maximum to 440 nm (21,22). Here, we used acidification to measure the regeneration of photoactivated blue cone opsin.

### **Fluorescence spectroscopy to study changes at the retinal binding site**

QuantaMaster 4 spectrofluorimeter (Proton Technology International) was employed to measure the fluorescence emission corresponding to retinal release and uptake processes. The excitation wavelength was 285 nm, and the emission wavelength was 335 nm, measuring 1 point per second for 2 s, followed by a 28 s pause (with a beam shutter to prevent sample photobleaching by the fluorimeter lamp). The excitation slit setting was 0.5 nm, and the emission was at 10 nm. In the case of green cone opsin, the Trp fluorescence from the binding pocket was measured by using 295 nm as excitation wavelength and 330 nm for emission wavelength. Fluorescence from the aromatic functional group was monitored over time, in the dark, until a steady baseline was obtained. Then the sample was photobleached for 30 s using a Dolan-Jenner MI-150 fiber-optic illuminator, and the change in fluorescence was recorded. For regeneration experiments, a 2.0-2.5-fold molar concentration of retinal over pigment was used, from a concentrated retinal stock in absolute EtOH, which accounts for 1% of the final protein sample volume.

### **Molecular Modelling**

Homology models of the dark-state and active forms of blue, green and red cone opsins were created using Modeller 9.16 (23) based on rhodopsin structures with PDB id 1GZM (24) and 3DQB (25) respectively. Acidic and basic residues were considered charged except Asp<sup>2.50</sup> (dark-state/active rhod, green and red), Glu178<sup>ECL2</sup> (dark-state rhod and blue), Glu<sup>3.28</sup> (active rhodopsin, green, red and blue), Glu<sup>3.37</sup> (dark/active rhodopsin) and Glu<sup>2.53</sup> (dark/active red and

green) which were taken protonated since they do not have a counterpart. Twenty-six crystallographic internal water molecules present in the inactive and active structures, were kept as part of the model. The dark-state model included a covalently bound retinal molecule. All systems were embedded in a pre-equilibrated cubic box containing 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphatidylcholine (POPC) lipid bilayer. The final systems had ~180 POPC molecules, ~16000 water molecules and a 0.2 M concentration of Na<sup>+</sup> and Cl<sup>-</sup> ions. All systems were energy-minimized and subsequently subjected to a 21 ns molecular dynamics (MD) simulation for equilibration, with decreasing positional restraints on protein coordinates. These restraints were released, and 500 ns of MD trajectory were produced at constant pressure and temperature. Simulations were run with the GROMACS 5.1.2 simulation package(26), using the AMBER99SBILDN force field as implemented in GROMACS and Berger parameters for POPC lipids. This procedure has been previously validated (27). Retinal parameters were taken from a previous work (18).

## RESULTS AND DISCUSSION

### Inactive and active state retinal binding sites of blue cone opsin

We generated molecular models of blue cone opsin at its dark (inactive) and active state conformations opsin (Fig. 1A and 1C, respectively) based on the homology with rhodopsin (see Methods). These models provide the structural framework to understand the process of chromophore regeneration of photoactivated blue cone opsin. For comparison purposes we also constructed analogous models of green (Fig. 1B and 1D) and red (not shown) cone opsins. These models reveal many analogies between the residues of the retinal binding pocket in rhodopsin and in the blue cone opsin, suggesting a similar activation mechanism. For instance, rhodopsin structures show that the counterion of Lys296<sup>7.43</sup>/SB is Glu113<sup>3.28</sup> in the dark state and Glu181<sup>ECL2</sup> in the opsin form. Both residues are conserved in blue cone opsin (Glu110<sup>3.28</sup> and Glu178<sup>ECL2</sup>), whereas red and green cone opsins lack Glu<sup>ECL2</sup> and have an alternative Glu residue at position 2.53 (Glu102<sup>2.53</sup>) near both Lys312<sup>7.43</sup> and Glu129<sup>3.28</sup>. Thus, the possible ionic interactions of Lys<sup>7.43</sup>/SB are with Glu<sup>3.28</sup> or Glu<sup>ECL2</sup> in rhodopsin and blue cone opsin, and with Glu<sup>3.28</sup> or Glu<sup>2.53</sup> in red/green cone opsins. We performed molecular dynamics (MD) simulations of rhodopsin and the three cone opsins starting from either the dark-state or the opsin forms (see Methods) and monitored the counterions in less than 3.5 Å to the SB (Fig. 1E). Blue opsin shows distance profiles compatible with the changes of counterion observed in the crystal structure of rhodopsin (Lys<sup>7.43</sup>/SB - Glu113<sup>3.28</sup> in the dark inactive state and Lys<sup>7.43</sup> - Glu181<sup>ECL2</sup> in the opsin form), but the simulations of the opsin state of both the red and green opsins indicate that Glu102<sup>2.53</sup> is the main counterion of Lys312<sup>7.43</sup> in the active form (Fig. 1E). This implies a different orientation of the Lys<sup>7.43</sup> side-chain in red/green cone opsins that might

have consequences in the functional properties of the photoreceptors. Moreover, the proximity between Glu<sup>3.28</sup>, Lys<sup>7.43</sup>, Glu<sup>2.53</sup> and Asp<sup>2.50</sup> enables the formation of a large cluster of internal water molecules that connects the four residues with the conserved Asn<sup>1.50</sup>, Trp<sup>6.48</sup> and Asn<sup>7.49</sup> (Fig. 1E). The size of the water molecule cluster is smaller in rhodopsin and blue cone opsin which both lack Glu<sup>2.53</sup>, a difference that may also have consequences in the transmission of the signal towards the cytoplasmic side where G protein activation takes place. Two particularities of the blue cone opsin structure are the absence of the highly conserved Asp<sup>2.50</sup> (it features Gly80 instead) and the presence of Tyr262 at position 6.48 instead of Trp (Trp is present at this position in most GPCRs including the other sensory opsins). These specific changes suggested that blue cone opsin might exhibit a distinct retinal binding mechanism than other visual pigments. However, our MD simulations of the blue cone opsin did not show effects that can be associated to these changes and suggest that Thr121<sup>3.39</sup> (Gly140<sup>3.39</sup> in the red/green cone opsins) compensates for the lack of Asp<sup>2.50</sup> by occupying a similar position in the network.

### **Chromophore regeneration of blue cone opsin**

The chromophore regeneration mechanisms from purified recombinant blue cone pigment can be analyzed in real-time using UV-vis and fluorescence spectroscopy by treating the photoactivated opsins with retinal analogs.

In the present study, blue cone opsin was illuminated using white light. Therefore, it was necessary to exclude the potential regeneration of available free opsin with retinal isomers other than ATR. Therefore, after measuring a dark state UV-vis spectrum, the sample was illuminated using white light, subsequently acidified and the corresponding spectra recorded. A band at 440 nm, in the difference spectrum between the acidified and illuminated spectra would indicate the presence of SB-linked regenerated species but no such band was observed (Fig. S1). This indicated that free opsin did not regenerate with any retinal isomer (other than ATR) that could have potentially been formed by illumination with white light.

The molecular changes at the active chromophore binding site of blue cone opsin can be measured by means of fluorescence spectroscopy. To this aim, the purified blue cone pigment sample in DDM detergent solution was illuminated using white light after obtaining a stable fluorescence baseline, and the fluorescent signal obtained was compared to that obtained for green cone opsin pigment (Fig. 2A). The sudden increase in fluorescence intensity suggests a fast release of the isomerized retinal upon photoactivation which is analogous to that observed for other cone pigments (18,20). Subsequently, 11CR was exogenously added at a concentration 2.5-fold over that of the purified pigment, immediately (Fig. 2B) or 15 min (Fig. 2C) after illumination and the resulting fluorescence changes recorded. In both cases we observed a decrease in fluorescence, a fact that suggested a binding process for blue cone opsin similar to that we previously observed for rhodopsin (18) with the same net reduction in fluorescence.

When adding 11CR immediately after photoactivation, the decrease in fluorescence intensity would reflect occupancy of the retinal binding pocket by the exogenously added chromophore (Fig. 2B). The two spectral components observed in Fig. 2B would reflect a fast fluorescence decrease as a result of photoactivation followed by the regeneration affecting Trp fluorescence quenching. The fluorescence changes detected when 11CR was added 15 min after photoactivation, might be the result of a conformational change with a slower kinetics which may occur specifically in the case of blue cone opsin. This conformational change occurs within the time lag of 15 min before 11CR addition and retinal entrance into the opsin binding pocket. We next validated the fluorescence results by means of parallel absorption UV-visible spectrophotometric measurements. The dark-state UV-visible spectrum of purified blue cone opsin in DDM showed its characteristic bands at 420 nm and 280 nm associated to chromophore-regenerated species and opsin, respectively. After measuring the dark-state spectrum of blue cone opsin, the sample was illuminated using white light, 11CR was immediately added and the regeneration was followed by continuously recording spectra for 60 min. The regeneration process of photoactivated blue cone opsin could not be followed unambiguously at 420 nm because the band corresponding to the exogenously added retinal masked the band of the regenerated blue cone opsin (Fig. 3A). Thus, we acidified the sample and followed the increase in absorbance at 440 nm indicative of SB-linked species that confirmed regeneration. A difference spectrum was obtained by subtracting the acidified and the regenerated spectra that could be compared to the difference spectrum between dark and illuminated spectra of the sample (Fig. 3A, inset). Comparison of these two difference spectra clearly shows a spectral shift to higher wavelength with equal amplitudes for the difference bands. Comparison with the dark-state acidified blue cone opsin (Fig. S2) also indicates the maximal extent of chromophore binding and regeneration. The regeneration experiment was also conducted by adding 11CR 15 min after illumination, mimicking the fluorescence experiment. Upon acidification at the end of the experiment, an absorbance increase at around 440 nm could be detected similar to that observed when adding retinal immediately after illumination (Fig. 3B). Difference spectra between acidified and regenerated conditions (compare Figs. 3A and 3B, insets) show a similar pattern in the two cases, suggesting retinal binding and chromophore regeneration.

### **A conformational change in photoactivated blue cone opsin impairs secondary 11CR uptake**

The regeneration kinetics of blue cone opsin cannot be determined from the UV-visible spectra because of the overlapping bands associated to free retinal and the regenerated opsin appearing in the same region of the spectrum. After the exogenous addition of retinal molecule, the

appearance of a 420 nm band, suggesting the regeneration of photoactivated pigment, is parallel to the decrease in 380 nm retinal band of the spectra. The absorbance change at 380 nm was monitored from 0 min to 60 min (Fig. S3) and fitted to a sigmoidal function (Fig. 3C): the obtained profile was similar to that previously observed for red and green cone opsins (20), though slower ( $t_{1/2} = 26.6$  min compared to 11.4 min and 13.1 min for red and green cone opsin, respectively). Blue cone opsin regeneration, with 11CR, was also conducted by adding retinal 15 min after illumination with no apparent change at 380 nm. The lack of absorbance change at 380 nm suggested absence of secondary retinal uptake by the photobleached opsin (Fig. 3D). This did not seem to perturb the regeneration at the primary retinal binding pocket (Fig. 3B).

### **A fine-tuning conformational change shifts the retinal analog specificity in free-state blue cone opsin at the photobleached state**

The regeneration of blue cone opsin was also tested with 9-*cis*-retinal (9CR) by exogenously adding this analog immediately or 15 min after illumination, in a similar way to the 11CR regeneration experiments above. The results showed that blue cone opsin regenerated with 9CR immediately after illumination (Fig. 4A) in a similar fashion to 11CR. Absorbance changes at 380 nm were monitored and showed a sigmoidal pattern (Fig. 4B) suggesting secondary retinal uptake. This result contrasts with the behavior of the other cone opsins that did not show secondary retinal uptake with 9CR (20). The same experiment was conducted at the post-bleached phase of blue cone opsin, i.e., 15 min after illumination and showed that 9CR could still bind to photoactivated blue cone opsin (Fig. 4C) similarly to 11CR (Fig. 3B). Surprisingly, secondary retinal regeneration rate was  $t_{1/2}$  28.8 min (Fig. 4D), that is, faster than the observed immediately after illumination. This result is in contrast with the 11CR case where no secondary binding site 15 min after photobleaching was observed.

Our results with purified blue cone opsin indicate that this opsin subtype can readily be regenerated *in vitro* with both 11CR and 9CR, and can undergo binding of a second retinal molecule other than that at the canonical primary binding pocket. The ability to bind the second retinal molecule is lost for 11CR (but not for 9CR) when retinal is added 15 min after illumination. This is likely due to a conformational change that renders blue cone opsin unable to accommodate the second 11CR.

In terms of sequence similarity, blue cone opsin is half way between red and green cone opsins (43%) and rhodopsin (46%). This is in contrast with the high similarity (96%) between red and green cone pigments (28). Furthermore, blue cone opsin evolved from an earlier taxonomical split with red and green cone opsins exhibiting a differential molecular basis for spectral tuning (12,29). Several key aromatic amino acids located in the retinal binding pocket are involved in

spectral tuning of visual pigments, including Trp123<sup>3,41</sup>, Phe205<sup>5,43</sup>, Phe209<sup>5,47</sup> and Tyr262<sup>6,48</sup> (Fig. S4) (3,30). These determine the optimal excitation and emission wavelengths to measure the maximal change in the fluorescence spectra of blue cone opsin, which were estimated to be 285 nm and 335 nm respectively (Fig. S5) when compared to excitation wavelength, 295 nm, and emission wavelength, 330 nm, used to study other opsins (18). A peculiar fluorescence signal decrease was observed for blue cone opsin, at a slow rate, after the initial increase which was not observed for green cone opsin (Fig. 2A) or red cone opsin (20). We believe that this could be due to a fine-tuning mechanism governing the conformational change that involves rearrangement of Tyr262<sup>6,48</sup> (Trp in red and green opsins and in rhodopsin (17)) in a slower fashion. This distinct conformational change, after isomerized retinal release, might be specific to blue cone opsin.

In our previous study, we have shown that opsins regenerate and remain functionally active even long time after photoactivation (18). In the present study, blue cone opsin was regenerated either immediately or 15 min after photoactivation. At these conditions, the stability of blue cone opsin in solution would even be presumably better than in our previous works with the other cone opsins where retinal was added 60 min after photocativation. Cone opsins are known for their faster dark adaptation compared to rhodopsin (31). Dark-state adaptation is favored by the more accessible retinal binding pocket with a relatively more open conformation (32). In terms of pocket accessibility, the hydroxylamine reactivity results (Fig. S6) showed that blue cone opsin is 10-fold more accessible than green cone opsin. This result suggests differences in the regeneration rates among the different cone opsin pigments but further experiments would be needed to prove this point.

We attribute the slower sigmoidal absorbance decrease at 380 nm to the uptake of a second retinal molecule by blue cone opsin, in agreement with our previous model for red and green cone opsins (20) and other studies (33-35). The lack of secondary retinal binding in the case of 11CR, when added 15 min after illumination, could be due to a conformational change that may fine-tune the spatial arrangement of amino acid residues at this secondary retinal site in this time frame. The measured half-life time for the kinetics of the secondary 9CR uptake is longer than that for 11CR (34.7 min versus 26.6 min), suggesting that 11CR binding is favored immediately after illumination. This interpretation is consistent with a previous study that analyzed the real time conformational dynamics of rhodopsin and showed a major conformational change in photoactivated rhodopsin involving larger movements of TMs 5 and 6 than those reported earlier (36).

The channeling hypothesis proposes that the retinal entrance/exit is controlled by receptor conformational changes that open/close the gates to the channel (34,37). Accordingly, upon SB hydrolysis, ATR may be initially trapped into the binding pocket, blocking the entrance of a fresh 11CR molecule (38). In our model, the secondary retinal uptake into regenerated cone

opsins may function as an 11CR buffer that may compensate for the fast decay of the photoactivated conformation of cone opsins. Hence, *in vivo*, the regeneration of photoactivated blue cone opsin (and the associated conformational change) would be similar to those found in the present study but with a faster kinetics due to the higher availability of retinal molecules in the membrane environment. The conformational differences upon illumination, in the case of the blue cone opsin, may be connected to the fact that this pigment has a chromophoric band with significant overlap with that of free retinal as opposed to green and red cone opsins that absorb at longer wavelengths. An alternative explanation may be that scarce blue cone pigments need slightly delayed physiological response in comparison to the more abundant red and green cone pigments.

The slower sigmoidal kinetics observed in Fig. 4B would suggest that the secondary retinal interactions with opsin reported in the present study could exert inhibitory effects on the exit of ATR from the protein. Though the conformational change observed after photoactivation (under our *in vitro* conditions) may not necessarily occur *in vivo*, any mutations at the retinal pre-binding site would possibly affect the binding of its natural chromophore to its canonical site. The use of chromophore analogs compensating the compromised natural chromophore interactions could be a potential strategy for novel therapeutic approaches similar to the application of a synthetic 9-*cis*-retinoid analog for Leber's congenital amaurosis, a visual disease in which 11-*cis*-retinal synthesis is inhibited or abolished (39).

Additional MD simulations (data not shown) of both rhodopsin and the blue cone opsin, with retinal bound to the proposed secondary binding site for red and green cone opsins (40), suggest that this alternate site may also exist in rhodopsin and in blue cone opsin because retinal molecules remain bound through the trajectory course. We suggest that this secondary binding site may be used by both 11CR and 9CR before reaching the canonical retinal binding pocket constituting a "membrane vestibule" similar to what has been proposed for the sphingosine-1-phosphate receptor 1 (40).

Overall, regeneration experiments of photoactivated blue cone opsin with retinal analogs, at different time points after photobleaching, suggest that a fine-tuned conformational change takes place for photoactivated blue cone opsin, that could involve helical rearrangements affecting the movements of one, or more, aromatic amino acids that would be responsible for the intensity changes observed in the fluorescence experiments and for the differences observed in the binding of different retinal analogs at the secondary site (Fig. 5). This phenomenon may have similarities with that observed for rhodopsin in its retinal binding site (18). Moreover, differences in intragenic epistatic interactions between the structurally related red, green and blue cone opsins (41,42) may justify the existence of the different functionally-relevant conformations reported in the present study.

## **AUTHORS CONTRIBUTION**

S.S. planned and conducted the experiments, discussed the results and wrote the main manuscript text. M.A.F.S. conducted the disulfide bond double-mutant experiments. M.M. and E.R. contributed to the discussion and to the final written version of the manuscript. M.J.R. and A.C. were responsible of the molecular modeling simulations and wrote the corresponding theoretical section. P.G. planned the overall experimental strategy and contributed to the discussion of the results and the writing of the manuscript.

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## FIGURE LEGENDS

**FIGURE 1.** Molecular models and MD simulations of the blue and green cone opsins. **(A, B)** Representative snapshots of the dark-state (inactive) blue and green cone opsins. **(C, D)** Representative snapshots of the active blue and green cone opsins. **(E)** Distance profiles between Lys7.43 and Glu3.28, GluECL2 and Glu2.53 ( $<3.5$  Å).

**FIGURE 2.** Fluorescence changes attributed to the chromophore binding site of blue cone opsin upon photoactivation and regeneration. **(A)** Fluorescence changes of the retinal binding site, presumably from Tyr6.48 and to some extent from Trp3.41, from the purified human blue cone pigment (solid line) before and after illumination were compared to the fluorescence changes of Trp<sup>6.48</sup> of purified green cone pigment (dotted line). **(B, C)** 2.5 fold 11CR to the concentration of the blue cone pigment was added immediately **(B)** and 15 min **(C)** after photoactivation and the fluorescence changes were recorded.

**FIGURE 3.** Photoactivated blue cone opsin shows regeneration with 11CR both immediately (0 min) and 15 min after illumination. **(A)** The UV-visible spectrum of blue cone pigment was measured in the dark (1), after illumination of the sample (2) and after immediate addition of 2.5-fold of 11CR, over pigment concentration, after illumination (3). The regenerated spectrum was recorded after 60 min (4), followed by sample acidification using 2N H<sub>2</sub>SO<sub>4</sub> (5). *(inset, A)* difference spectra were plotted by subtracting the dark minus illuminated spectra (6), and the acidified and last regenerated spectra (7), respectively. **(B)** Same experiment as in **(A)** but the addition of 11CR was carried out 15 min after illumination. **(C)** After the addition of 11CR in both the cases (immediately and 15 min after photoactivation), the regeneration was followed at 380 nm for 60 min. The decrease in absorbance at 380 nm plotted versus time, and fit to a sigmoidal curve **(D)** The same was performed during the 11CR regeneration experiment in which the retinal was added 15 min after illumination. In this case data did not fit to a sigmoidal curve but showed a linear dependence.

**FIGURE 4.** Regeneration and secondary retinal uptake of blue cone opsin with 9CR at 0 min and 15 min after photoactivation. **(A)** 9CR regeneration experiment was performed with blue cone opsin, the retinal was added immediately after photoactivation and the difference spectra were obtained by subtracting the dark minus illuminated spectra (1), and also by subtracting the acidified minus the regenerated spectra (2). **(B)** The absorbance changes observed over 60 min at 380 nm were plotted and fit to a sigmoidal curve. **(C, D)** The same was carried out but 15 min after photoactivation, and the corresponding difference spectra **(C)**, and secondary retinal uptake sigmoidal curve were obtained **(D)**.

**FIGURE 5.** Schematic representation of the activation and chromophore regeneration processes for blue cone opsin. After the purified blue cone pigment (blue shape) with 11CR (orange shape) is illuminated, 11CR isomerizes to ATR (green shape) and rapidly activates the receptor and, subsequently, the G protein (gray shape). The associated conformational changes open a channel that is used by retinal to exit or enter (43). After retinal leaves, the opsin form is in equilibrium between different states (represented by the light blue shapes) that allow entrance of both the 11CR and the 9CR via the putative “membrane vestibule”. The chromophore can regenerate immediately ( $t = 0$  min) and 15 min after photoactivation. After regeneration the retinal channel progressively closes and goes back to the fully closed state. Our data suggests the existence of a secondary retinal site in the blue cone opsin that is available for both 11CR and 9CR at  $t = 0$  min, but not for 11CR long time after activation ( $t = 15$  min), probably due to the conformational changes that do not affect the binding of 9CR.

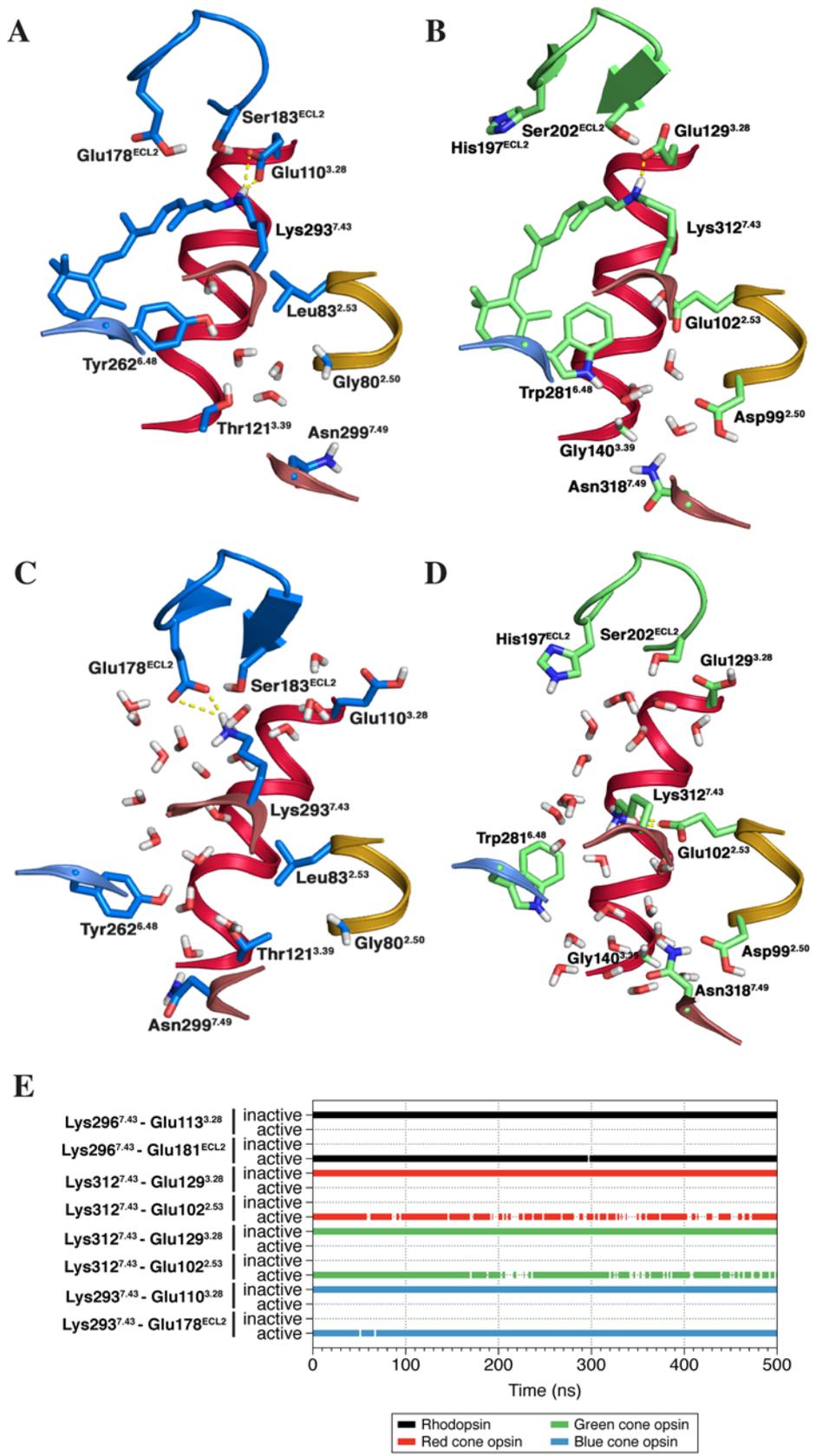
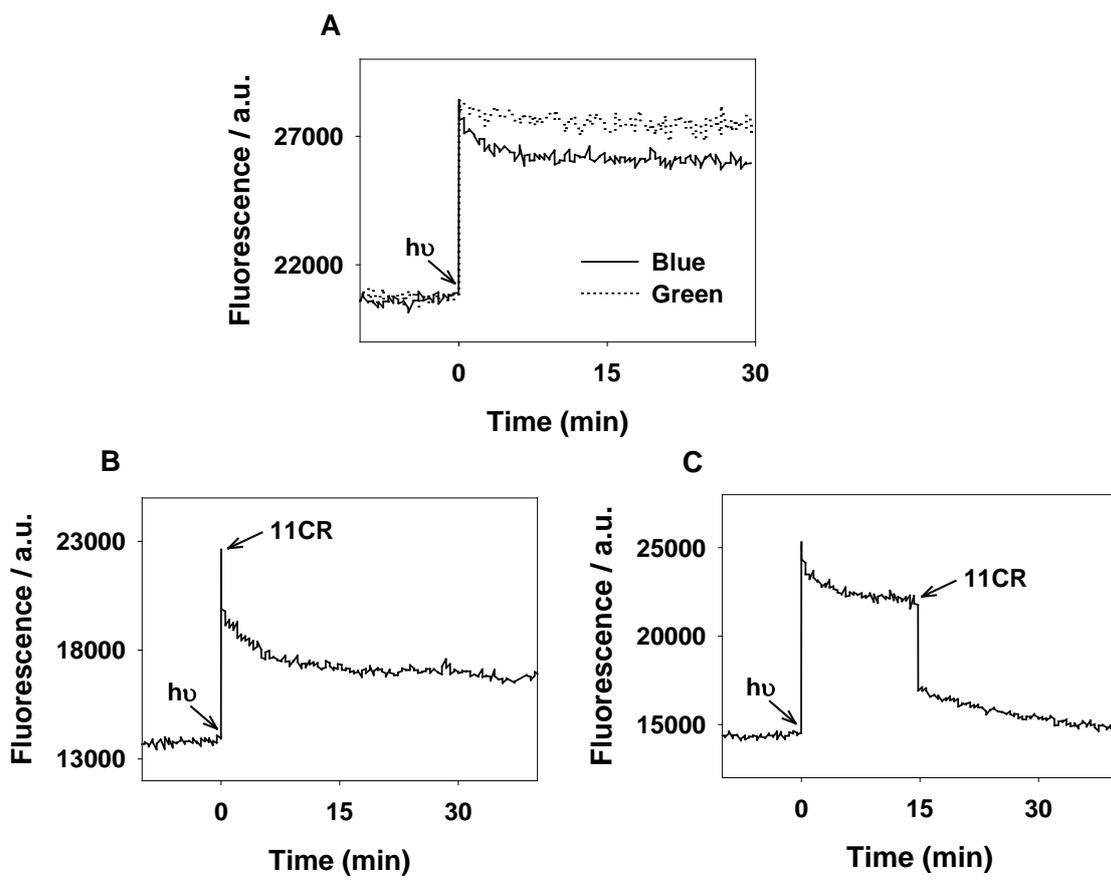


FIGURE 1



**FIGURE 2**

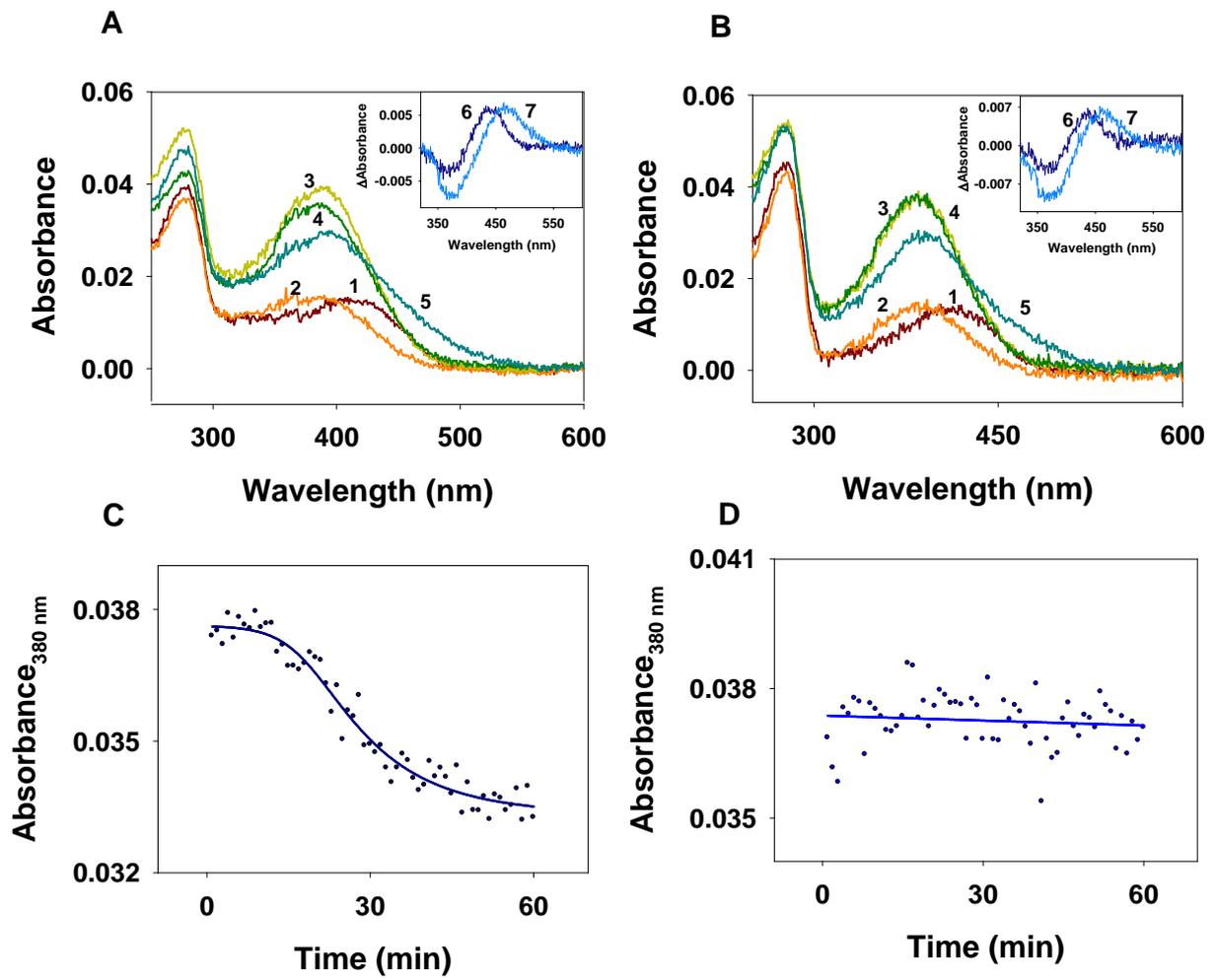


FIGURE 3

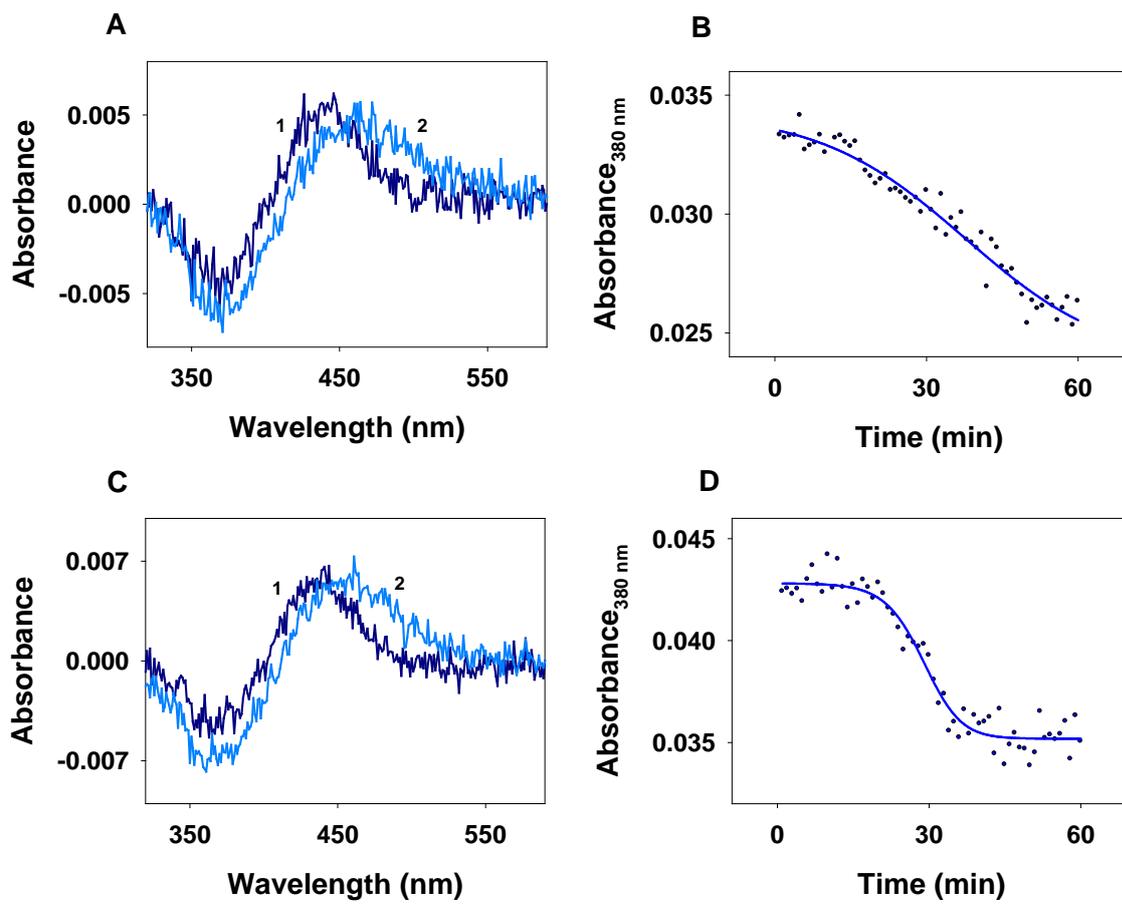
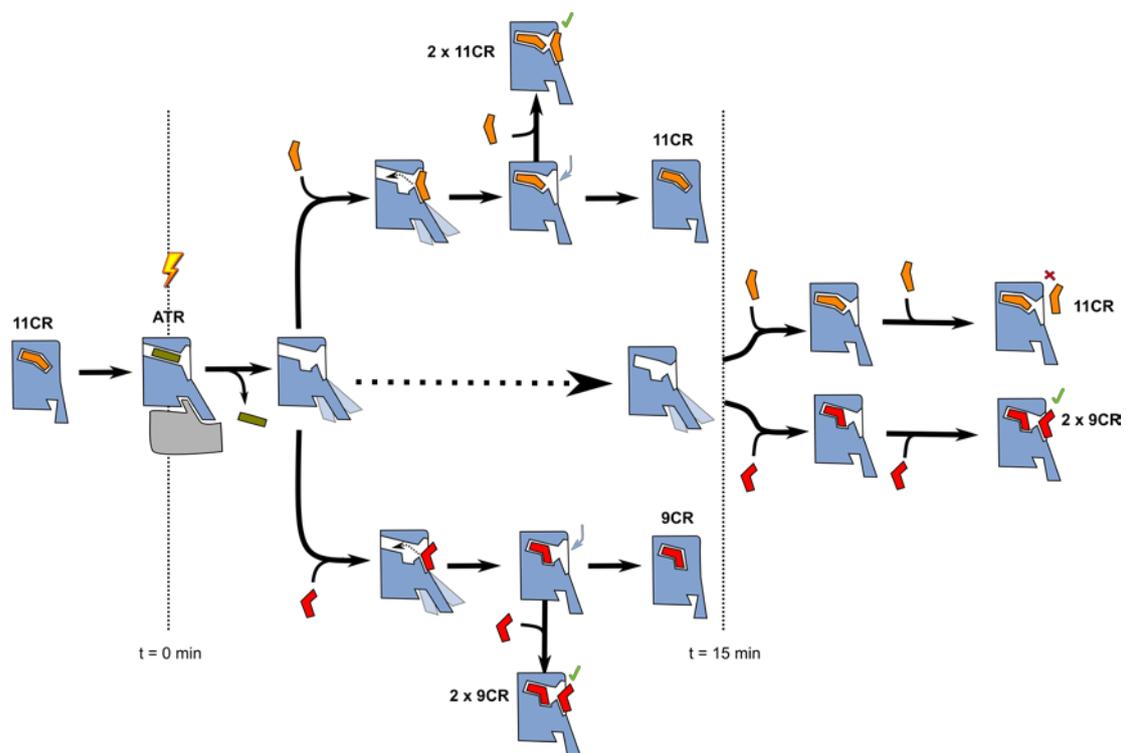


FIGURE 4



**FIGURE 5**